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Cloning and Identification of Single Chain Variable Fragment Recombinant Antibodies to Botulinum Neurotoxin A

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Technical Report
DRES TR 1999-190

December 1999

National Defence / Défense nationale

Canada

DEFENCE RESEARCH ESTABLISHMENT SUFFIELD
RALSTON, ALBERTA

TECHNICAL REPORT 1999-190

CLONING AND IDENTIFICATION OF SINGLE CHAIN VARIABLE FRAGMENT RECOMBINANT ANTIBODIES TO BOTULINUM NEUROTOXIN A

by

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ACKNOWLEDGEMENTS

The authors would like to thank Fay Schmaltz for technical assistance.

ABSTRACT

Balb/C mice were inoculated with botulinum toxoid followed by up to 2.0 x 10^5 LD_{50} of botulinum A neurotoxin (BoNT/A). Splenomic RNA was isolated from hyperimmune mice and used to prepare a cDNA library. DNA fragments encoding recombinant single chain fragment variable (scFv) antibodies to BoNT/A were cloned using an M13 phage display system. Individual clones were obtained by immunoaffinity selection using immobilized BoNT/A and screened by enzyme-linked immunosorbant assay (ELISA). Western blots were then used to confirm the reactivity of these clones. One hundred and twenty-two clones were obtained that reacted to the botulinum toxin A used in the initial inoculations. These were then tested by ELISA using highly purified BoNT/A, and forty-eight were found to be reactive.

EXECUTIVE SUMMARY

Title

Mah, D.C.W., Pon, J.K., Masri, S., Fulton, R.E., Monette, P.L., and Nagata, L.P. Cloning and Identification of Single Chain Variable Fragment recombinant antibodies to Botulinum Neurotoxin A, Technical Report 1999-066, November 1999.

Introduction

Recent advances in recombinant antibody technology now provide a potential source of specific antibodies which is economical, stable and versatile. The M13 phage display system is one of the methods for producing recombinant antibody libraries. In this system, the antigen recognition domains of the light and heavy antibody chains are cloned, joined together via a peptide linker, and expressed on the surface of the M13 phage. By immobilization of the antigen on a solid support, the recombinant phage which are reactive to the antigen can be selected. In this paper, we discuss the construction and characterization of recombinant antibody libraries to botulinum toxin A.

Results

This report describes the cloning and characterization of murine and human recombinant antibodies using the M13 phage display system from a collection of 122 clones selected by binding activity to botulinum toxin complex. Of these, 48 were found to be reactive to highly pure botulinum A neurotoxin. Twenty-three of the scFv clones have been well characterized and a few of the most reactive will be selected for high level expression in the near future.

Significance of Results

Immunoidentification and passive immunotherapeutic techniques are dependant upon the availability of antibodies. Conventional antibodies are produced *in vivo* in animals i.e., polyclonal antibodies, or *in vitro* by culturing of hybridoma cells which secrete monoclonal antibodies. More recently, it has become possible to produce recombinant antibodies e.g., scFv antibodies, through application of the techniques of recombinant DNA technology. For use as immunodiagnostic and immunotherapeutic reagents, recombinant antibodies have a number of potential advantages over conventional antibodies, including enhanced sensitivity, specificity, and reproducibility. In addition, recombinant antibody techniques can be used to rescue important monoclonal antibodies from unstable hybridomas. Of particular importance is the fact that antibody structure and function can be controlled at the gene level through manipulation of genes, resulting in, for example, antibodies with enhanced antigen binding properties, or antibodies fused genetically to other proteins e.g., reporter molecules. Furthermore, "humanized" antibodies which are safe for therapeutic administration to humans can be cloned. Such antibodies can be administered to humans without concern for "serum sickness," resulting from inoculation of animal proteins, or for the presence of adventitious agents, which are potential contaminants of extraneous human immunoglobulins. The use of scFv antibodies also addresses problems of producibility and availability, as the scFv product can be expressed in high volume in alternative hosts and purified therefrom.

This report describes the cloning and characterization of a ScFv antibody library to botulinum neurotoxin A. The findings demonstrate the functionality of ScFv antibodies cloned directly from mouse spleen RNA, thus bypassing the need for hybridoma cell production with its inherent instability of antibody expression. Antibodies to botulinum A neurotoxin have been identified, which may prove useful in detection/identification assays and which could be engineered into useful products for protection/treatment of botulinum A intoxication.

Future Goals

Future research focuses on the development of high-level expression systems for the production of recombinant antibodies. A collaborative effort was recently setup with Dr. Rick Titball, Chemical Biological Defense Establishment (Porton, UK), to further characterize the recombinant antibody clones and libraries to botulinum toxin A for neutralization and protection. As well, the development of chimeric antibodies, in which the antigen recognition domain is derived from mouse antibody and the functional (Fc) domain is derived from human antibody, could prove useful in protection/treatment using immunotherapy. Chimeric antibodies have the advantage of reducing the risk of developing adverse human anti-mouse antibodies and serum sickness.

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INTRODUCTION

Botulinum neurotoxin (BoNT) is a deadly protein toxin, produced under anaerobic conditions, by strains of *Clostridium botulinum* (1, 2). Seven serotypes of the BoNT, classified A through G, are recognized, but only serotypes A, B and E are clinically important agents of human botulism (1, 3, 4). The various serotypes proteolytically cleave specific proteins which are involved in synaptic vesicle docking and thus disrupt cellular communication at the neuromuscular junction (5). Neurotoxicity is caused by the irreversible blockage of acetylcholine neurotransmitter release at the neuromuscular junction (1, 6, 7). Serotype A can be purified as a 900-kDa complex (BoNT/A-HA) consisting of a toxic component, botulinum neurotoxin serotype A (BoNT/A), and a nontoxic component, hemagglutinin (HA) (8, 9, 10).

The BoNT consists of a C-terminal 100-kDa heavy chain (H subunit) linked through a disulfide bond to an N-terminal 50-kDa light chain (L subunit). These are generated from the proteolytic cleavage of a 150-kDa haloprotein (1, 4). Three 50-kDa functional domains have been recognized in the neurotoxin; from C-terminus to N-terminus, they are: a binding, a translocation, and a catalytic domain (11, 12, 13). The H subunit binds to specific ganglioside receptors on the target neuronal cells (14, 15) and the protein passes across the membrane (16). The L subunit, which contains a zinc metalloprotease activity, then cleaves proteins involved in synaptic vesicle docking (5, 7, 11, 17). The role of the protein subunits of the nontoxic component, both the nontoxic agglutinating proteins (18, 19, 20) and the nontoxic nonagglutinating proteins (14, 21), is not well understood. Recent experiments, however, suggest that the nontoxic components and the toxic components of the 900-kDa complex may protect each other from pH extremes and gastrointestinal proteases (8).

Botulism can be diagnosed by clinical symptoms, but differentiation from other diseases can be difficult. Some laboratory tests can be used to confirm the clinical diagnosis of botulism by demonstrating the presence of toxin in the serum of the patient. Currently, the most sensitive and widely used method for detecting toxin is the mouse neutralization test, a procedure which takes 48 hours to complete.

Clearly, there exists a need for more rapid and sensitive tests for the detection of BoNT/A. The production of BoNT/A-specific antibodies for the efficient detection of this hazardous environmental agent is an essential first step in the development of a more rapid and sensitive diagnostic procedure. Neutralizing antibodies to BoNT/A could also provide an efficient passive immunity to endangered individuals. In order to address these needs, we have engineered some antibodies that recognize BoNT/A in ELISA. This paper reports on the cloning and selection of recombinant mouse single chain variable fragment (scFv) antibodies to BoNT/A.

MATERIALS AND METHODS

Botulinum toxoids and toxins

Botulinum pentavalent ABCDE toxoid (BoTd/P), 10% purity, was obtained from the Center for Disease Control, Atlanta, GA. BoNT/A [(1 mg/ml; LD_{50} = 3 X 10^{-5} - 1.5 X 10^{-4} μg protein (by intraperitoneal (IP) injection)] was obtained from Wako Chemicals (Richmond,VA). Botulinum A toxoid (BoTd/A) was prepared by incubating BoNT/A in 3% formaldehyde at 37°C for 10-14 days and then dialyzing extensively against phosphate buffered saline, pH 7.2. Highly purified (99% pure) BoNT/A was obtained from the Food Research Institute, University of Wisconsin, Madison, WI.

Vaccination of mice

Three-week-old Balb/C female mice were immunized to BoNT/A using a two-phase vaccination schedule. In phase one, the mice received five IP inoculations, at 10-14 day intervals, of 20 μg of BoTd/P or BoTd/A, supplemented with 20 μg of muramyl dipeptide adjuvant. In phase two, mice received four IP inoculations with increasing amounts of BoNT/A toxin: 10 ng (300 LD_{50}), 100 ng, 500 ng, and 5 ug. The toxin was, in each case, supplemented with 20 ug muramyl dipeptide and the interval between inoculations was as in phase one. The mice were sacrificed and their spleens were removed about 10 days after the final inoculation.

Antisera titers at that time were between 1:1000 and 1:10000, based on immunodot blots using biotinylated BoNT/A.

RNA extraction

Four spleens were pooled for each of two extractions. The tissues were homogenized in a solution of guanidinium isothiocyanate by use of a Brinkmann homogenizer. RNA was further purified by isopycnic density gradient centrifugation on cesium chloride (22). Yields of 600-650 μg of spleen total RNA were obtained. The quality of the RNA was assessed by electrophoresis in 1% agarose gels. Distinct bands for the 28S and 18S ribosomal RNA species were observed and the RNA was deemed to be of acceptable quality for reverse transcription.

Reverse transcription and PCR amplification

Spleen total RNA (10-20 μg per reaction) was used as template for reverse transcription. Primer mixtures from a Recombinant Phage Antibody System - Cloning Kit (Pharmacia Biotech Inc., Montreal, QU) were then used in PCR (94°C/1 min; 55°C/2 min; 72°C/2 min; 30 cycles) to amplify cDNA sequences corresponding to the V_H region and the V_L region of mouse IgG Kappa. DNA fragments were resolved by electrophoresis in 0.8% low melting-point agarose (Seakem, FMC) and purified by phenol/chloroform extraction followed by ethanol precipitation. The amount of DNA in each fragment preparation was estimated by dotting aliquots onto agarose plates containing 0.1 μg/ml ethidium bromide and comparing their fluorescence to that of DNA standards. Equal amounts (0.1-0.2 μg) of gel-purified V_H and V_L DNA were annealed with a 100 base pair (bp) linker-primer $(Gly_4Ser)_3$. Full-length scFv gene fragments were then generated with *Taq* DNA polymerase (Perkin Elmer, Foster City, CA) (94°C/1min; 63°C/4 min; 7 cycles). Buffer conditions were optimized and full-length scFv DNA fragments were amplified (94°C/1 min; 55°C/2 min; 72°C/2 min; 30 cycles) using primers corresponding to *Sfi* I and *Not* I restriction sites. The scFv DNA fragments were purified after electrophoresis on 0.8% low melting-point agarose and then digested with the

restriction endonucleases *Sfi* I and *Not* I. The restriction enzyme-digested fragments were purified from low melting-point agarose gels and ligated to the phagemid pCANTAB 5 at the *Sfi* I to *Not* I site.

Transformation of TG1 E. coli

Competent *E. coli* TG1 cells were prepared as described by Hanahan (23). Ligated DNA (5-50 μl) was placed in a sterile tube and chilled for 5 min. One hundred μl of chilled competent TG1 cells was added to the DNA and the mixture was incubated for 30 min on ice. LB broth supplemented with 2% glucose was then added to each tube (0.9 ml per tube) and the transformation mix was incubated at 37°C for 1 h, with shaking at 150 rpm. Three hundred μl of the mix was then spread onto each of several 100 mm plates containing LB agar supplemented with 100 μg/ml ampicillin and 2% glucose and the plates were incubated overnight at 37°C. A recombinant pCANTAB 5 scFv library was then obtained by pooling the transformant colonies on the plates.

Phage rescue of transformed cells

Phage rescue with M13KO7 helper phage results in the production of recombinant phage containing a copy of the phagemid DNA encoding a specific antibody scFv gene. The recombinant phage will display one or more copies of the recombinant antibody as fusion proteins on their tips. For phage rescue, 100 μl of an overnight culture of the transformed *E. coli* TG1 (and thus of the recombinant antibody library) was added to 1 ml of 2X YT broth supplemented with 100 μg/ml ampicillin, 2% glucose and 3 X 10^{10} pfu/ml M13KO7 (Pharmacia Biotech Inc.). This culture was incubated at 37°C for 2 h, with shaking at 250 rpm. The cells were pelleted by centrifugation for 10 min at 5000 X *g* and the supernatant was discarded. The cell pellet was resuspended in 5 ml of 2X YT broth supplemented with 100 μg/ml ampicillin and 50 μg/ml kanamycin, and incubated overnight at 37°C, with shaking at 250 rpm. The cells were then pelleted by centrifugation for 15 min at

5000 X *g*. Recombinant phage were collected by decanting the supernatant into a sterile 5 ml tube.

Immunoaffinity selection of recombinant phage

As the phage-displayed antibody fragments are fully functional and will bind antigen, they can be affinity-selected using the target antigen. This process ultimately results in enrichment for those phage which display antigen-positive scFv on their surface and contain the gene coding for antigen-specific scFv expression. Affinity selection was conducted in Falcon polyvinyl carbonate (PVC) 96-well round-bottom plates (Fisher Scientific, Nepean, ON). Fifty μl of BoNT/A antigen (6 μg/ml) was added to each of four wells containing 50 μl of 2X coating buffer (0.1 M sodium carbonate, pH 9.2). The plates were incubated overnight at 4°C. Unbound antigen was removed from the wells by two rinses with sterile phosphate buffered saline containing 0.05% Tween 20 (PBST). One hundred and fifty μl of blocking buffer (sterile 5% skim milk in PBS) was added to four wells containing antigen and to one well with no antigen. The plates were incubated for 2 h at room temperature. The phage suspension was pre-adsorbed with 5% skim milk prior to affinity selection. Pre-adsorption was performed by adding 150 μl of the rescued phage suspension into the blocked well which contained no antigen and incubating for 1 h at room temperature. Following the pre-adsorption step, the phage suspension was transferred to the first of four wells containing bound antigen. The plate was incubated for 1 h at room temperature, after which, unbound phage was removed from the well. The well was then rinsed ten times with PBST and ten times with PBS. Bound phage was eluted from the well by the addition of 150 μl of 100 mM triethylamine and transferred to a microfuge tube. Eluted phage was neutralized with 75 μl of sterile 1 M Tris, pH 7.2. One hundred and fifty μl of the eluted, neutralized phage was added to the second well containing bound antigen, and the above process was repeated. The rescued phage was subjected to four such consecutive cycles of affinity selection.

Isolation of individual clones expressing a scFv

E. coli TG1 cells were grown overnight at 37°C. Two hundred μl of the overnight culture was added to a 5 ml tube containing 20 μl of eluted phage from the affinity selection step. One tube was prepared for phage eluted from each of the four wells used in affinity selection (described above). The tubes were incubated at 37°C for 30 min, with shaking at 150 rpm. The contents were then spread onto 82 mm Luria-Bertani (LB) agar plates supplemented with 100 μg/ml ampicillin and 2% glucose; the plates were incubated at 37°C overnight. Individual colonies isolated from these plates thus represented individual recombinant clones. Similarly, an aliquot (150 μl) of the overnight culture of TG1 cells was added to each well previously used in affinity selection. The wells were incubated at 37°C for 30 min, with shaking at 150 rpm. The contents of the wells were then spread on LB agar as above, in order to obtain individual colonies. Single colonies from each of these eight plates were inoculated into 1.5 ml LB broth supplemented with 100 μg/ml ampicillin and 2% glucose and cultures were incubated at 37°C overnight.

Phage rescue from isolated colonies (individual recombinant clones)

Cultures obtained by growing individual colonies (as described above) were inoculated (200 μl) into 1 ml of 2X YT broth containing 100 μg/ml ampicillin, 2% glucose, and 3 X 10^{10} pfu/ml of M13KO7. These cultures were incubated for 2 h at 37°C, with shaking at 150 rpm. The cells were pelleted by centrifugation at 5000 X *g* for 10 min and the supernatant was discarded. The cell pellet was resuspended in 1 ml 2X YT broth containing 100 μg/ml ampicillin and 50 μg/ml kanamycin. The suspension was incubated at 37°C overnight, with shaking at 250 rpm. The cells were then pelleted by centrifugation at 5000 X *g* for 15 min and recombinant phage was collected by decanting the supernatant into a sterile 5 ml tube.

Identification of reactive clones

An ELISA, designed to detect recombinant phage antibody bound to antigen-coated wells, was conducted to determine which of the individual rescued

recombinant phage produced reactive anti-BoNT/A antibody. Falcon PVC 96-well round-bottom plates were used in this assay and samples were applied in duplicate. Fifty μl of 2X coating buffer (0.1 M sodium carbonate, pH 8.2) was added to each of the assay wells, followed by 50 μl of 6 μg/ml BoNT/A. Two wells were used as negative controls; one well received 50 μl PBS and the other well received 50 μl of 10 μg/ml BSA. Three wells were used as positive controls; one well received 50 μl of 2 μg/ml sheep anti-M13 antibody (Pharmacia Biotech Inc.), another well received 50 μl of M13KO7 bacteriophage (Pharmacia Biotech Inc.) at a concentration greater than 5 X 10^{10} pfu/ml, and the third well received BoNT/A antigen. The perimeter wells received only 50 μl of 2X coating buffer plus 50 μl of PBS. The plate was incubated at 4°C overnight. The plate was then emptied and the wells were rinsed twice with PBST. Blocking buffer (5% skim milk) was then added to all wells (150 μl/well) and the plate was incubated at room temperature for 2 h. The blocking buffer was then discarded and 30 μl of fresh blocking buffer was added to each well. Rescued individual recombinant phage from 25 isolated colonies were added to separate wells, 120 μl/well. The five control wells each received 120 μl of M13KO7 helper phage; alternatively, these wells received 120 μl of D12-3 phage, a phage standard displaying a scFv against botulinum toxoid. The perimeter wells received 120 μl PBS. The plate was incubated at room temperature for 1 h. The plate was then emptied and the wells were rinsed with PBST. Sheep anti-M13 horseradish peroxidase (HRP) conjugate diluted 1:2000 in blocking buffer was added to the wells, 100 μl/well, and the plate was incubated at room temperature for 1 h. The conjugate was then discarded, and the plate was rinsed once with PBST and once with PBS. Substrate [(2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid)] (ABTS) was added to the wells, 150 μl/well, and the plate was incubated at room temperature for 1 h and monitored for color development.

Confirmation of clone reactivity: method 1

Clones which appeared to be producing the desired antibodies, based on ELISA, were further tested for their ability to immunoprecipitate biotinylated

BoNT/A. BoNT/A was biotinylated in accordance with procedures provided with a biotinylation kit purchased from Amersham, Arlington Heights, IL. Selected recombinant M13 (rM13) phage were incubated with biotinylated BoNT/A at room temperature for 1 h, and then with streptavidin-coated beads (Pierce, Rockford, IL) for 1 h. Immunoprecipitates were collected by centrifugation and washed three times with 0.5% Nonidet-EDTA (NET) (Sigma, Oakville, ON) buffer. Bound proteins were released from the precipitate by the addition of 2X lysis mix (Laemmli Sample Buffer, Bio-Rad, Mississauga, ON). Samples of the released proteins were analyzed by electrophoresis on 12% SDS-PAGE gels, followed by Western blotting. The gels were electroblotted using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad, Mississauga, ON) onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad). The membranes were incubated with blocking buffer, and then exposed to sheep anti-M13 HRP conjugate (Pharmacia Biotech, Uppsala, Sweden). Bands were visualized using an enhanced chemiluminescence (ECL) substrate (Amersham).

Confirmation of clone reactivity: method 2

Clones which appeared to be producing the desired antibodies, based on ELISA, were tested by a second method for their ability to immunoprecipitate biotinylated BoNT/A. Selected rM13 phage were incubated with biotinylated BoNT/A at room temperature for 1 h, then with rabbit anti-M13 antibodies (Pharmacia Biotech) for 1 h, and finally with beads bearing immobilized protein G (Pharmacia Biotech). Immunoprecipitates were collected and washed as described above. Bound proteins were released and analyzed as above, except that streptavidin-alkaline phospatase (AP) conjugate (Pierce) was used instead of sheep anti-M13 HRP conjugate. ECL detection was performed using CSPD^{R} substrate (Bio/Can, Mississauga, ON).

Confirmation of clone reactivity: method 3

Clones which appeared to be producing the desired antibodies, based on ELISA, were further tested by Western blotting for their ability to bind to BoNT/A.

A 15% SDS-PAGE, 0.75 mm thick and 8 cm wide, was used along with a 5% SDS-PAGE stacking gel, in a Bio-Rad Mini-gel apparatus (Mississauga, ON). Wells were prepared using a two-well comb. One well, for the protein molecular weight markers, was 0.5 cm wide; the other well, for the BoNT/A sample, was 7 cm wide. The BoNT/A antigen sample (100 μl; 0.15 μg/ml) was mixed with an equal volume of 5X sample buffer (250 mM Tris-HCl, pH 6.8, 10% SDS, 0.5% bromophenol blue, 50% glycerol, 3.6 M 2-mercaptoethanol). The mixture was heated at 100 °C for 5 min and applied to the sample well. Rainbow markers (10 μl) (Amersham) were added to an equal volume of 5X sample buffer, and the mixture was heated at 100°C for 5 min and then applied to the sample well. Electrophoresis was conducted at 200 V for 54 min. The gel was then equilibrated for 20 min in transfer buffer (39 mM glycine, 48 mM Tris base, 0.037% SDS, 20% methanol), as was a nitrocellulose membrane. Electroblotting was then conducted at 100 V for 1 h, using a Trans-blot SD Semi-dry Transfer Cell (Bio-Rad). The lane containing the molecular weight markers was cut from the nitrocellulose membrane. The remainder of the membrane was then cut into 0.5 cm strips parallel to the marker lane. The strips were numbered, then 10 μl of 10 μg/ml bovine serum albumin (BSA) was spotted at the top of each strip. The strips were incubated with blocking buffer at room temperature for 2 h, with shaking at 150 rpm. Each strip was then incubated at room temperature for 1 h with 2 ml of selected recombinant M13 in 5% blocking buffer. The strips were then rinsed several times with PBST and incubated at room temperature for 1 h in a 1:2000 dilution of rabbit anti-M13 HRP conjugate in blocking buffer, with shaking at 150 rpm. The conjugate was discarded and the strips were rinsed several times with PBST and then twice with PBS. Lumi-Glow chemiluminescent substrate (Life Technologies, Burlington, ON) was then applied to the strips. After a 5 min incubation at room temperature, the strips were placed on a sheet of plastic wrap and exposed to Kodak Biomax MR X-ray film (Amersham). After chemiluminography, the strips were placed in stripping buffer (0.1% SDS, 0.05% Tween 20 in PBS) and incubated for 30 min at 37°C, with shaking at 250 rpm. After washing three times in PBS, strips could be stored in PBS and used a second time.

Plasmid purification and restriction enzyme digestion

Plasmids were isolated from 1.3 ml each of selected recombinant M13 phage by an alkaline-lysis procedure (24). Plasmid DNA (0.2 μg) was digested with *Hind* III and *Eco*R I (Pharmacia Biotech Inc.), 2.5 U each, for 1 h at 37°C. Digestion products were analyzed on a 1% agarose gel and stained with ethidium bromide.

RESULTS

Construction of scFv libraries

Spleen total RNA from eight vaccinated Balb/C mice was reverse transcribed and the resulting cDNA amplified by PCR, using appropriate primers, to generate DNA fragments corresponding to V_H and V_L transcripts. Amplification of the V_H and V_L DNA fragments by PCR yielded the predicted 340 bp and 325 bp products, respectively (Fig. 1). The V_H and V_L DNA fragments, joined through a linker, were amplified by PCR to generate 750 bp scFv gene fragments (Fig. 2). The scFv DNA fragments were further amplified using two primers containing *Sfi* I and *Not* I restriction sites, respectively. The resulting products were then digested with *Sfi* I and *Not* I and ligated to pCANTAB 5 phagemid vector digested with the corresponding restriction enzymes. Bacterial phagemid clones obtained by transformation of *E. coli* TG1 with ligated DNA were selected on LB agar supplemented with ampicillin and glucose. Selected clones were then pooled to form a library. Thirteen such libraries, with a complexity of 10^5-10^6, were obtained from six preparations of scFv DNA fragments. Phage rescue with M13KO7 was then utilized to study the scFv phage libraries.

Immunoaffinity selection of recombinant phage

M13 phage containing recombinant antibody genes and displaying scFv antibodies specific to BoNT/A were isolated by immunoaffinity chromatography ("biopanning") using BoNT/A immobilized in the wells of PVC plates. Reactive clones were selected and their reactivity further evaluated by ELISA.

Evaluation of reactive clones

Four thousand clones, isolated as above by immunoaffinity chromatography, were screened by ELISA using BoNT/A (Wako Chemicals) (Fig. 3). Of these, 122 (3%) gave positive ELISA results. Ninety-six of the 122 clones were then further screened by ELISA, using highly purified BoNT/A (Food Research Institute). Forty eight of the 96 clones (50%) gave positive ELISA results. The 29 most reactive clones are listed in Table I.

Confirmation of clone reactivity

Three methods were used to further evaluate the reactivity of the clones which gave positive ELISA results. In the first method, selected clones were allowed to bind to biotinylated BoNT/A, and the immune complexes were precipitated with streptavidin-coated beads. Bound proteins were then released from the precipitate and analyzed by SDS-PAGE and Western blotting, using sheep anti-M13 HRP conjugate (Fig. 4). Of 10 clones tested, four were reactive and five were highly reactive. The tenth clone, designated D12, was very highly reactive (Table I). In the second method, selected clones were allowed to bind biotinylated BoNT/A, as described above. Rabbit anti-M13 antibodies were then added, followed by protein G-coated beads. Bound proteins were then released from the immunoprecipitated complexes and analyzed by SDS-PAGE and Western blotting, using a streptavidin-AP conjugate (Fig. 5). Of 11 clones tested, four were reactive and seven were highly reactive (Table I).

Thus, confirmation was obtained that the ELISA screening procedure identified clones which produced antibodies that bound to BoNT/A. The confirmation methods were distinct. The first method relied on streptavidin-biotin avidity for the capture of the recombinant M13. The second method was based on the affinity of rabbit anti-M13 antibodies for M13 proteins. The ability of the clones to bind to BoNT/A was also tested more directly by a third method, as follows. BoNT/A was subjected to SDS-PAGE and electroblotted onto nitrocellulose membranes. Western blots were then performed, using selected recombinant M13

clones as primary antibody and rabbit anti-M13 HRP conjugate as secondary antibody. All clones tested reacted with the BoNT/A.

Size distribution of the scFv inserts

The size distribution of the scFv inserts in the recombinant M13 was determined by subjecting purified phagemid DNA to restriction enzyme digestion with either *Hind* III and *Bam*H I, or *Sfi* I and *Not* I. The predicted size of the *Hind* III/*Bam*H I-digested scFv-containing DNA fragments is 1409 bp, and that of the *Sfi* I/*Not* I-digested scFv-containing DNA fragments is 745 bp (Fig. 6). Ninety eight of the 122 clones which gave a positive ELISA reaction with Wako BoNT/A antigen were digested with *Hind* III/*Bam*H I, and the digestion products were analyzed on agarose. The scFv inserts in these clones ranged in size from less than 200 bp to 1300 bp (Fig. 7a). The majority (68%) of these clones had inserts 401-800 bp in size. About 20% had much smaller inserts, less than 200 bp in size. The 29 most reactive clones (Table I) were also digested with *Sfi I/Not* I and their products analyzed as above (Fig. 7b). Sixty nine percent had inserts 401-800 bp in size. It was interesting to note that, of the most reactive clones, many had inserts 500 bp in size.

DISCUSSION

The results of our study clearly demonstrate the validity of the scFv approach for the preparation of antibodies to be used in the detection of BoNT/A. Toxoid was used in phase one of our vaccination schedule to develop anti-BoNT/A immunity in mice prior to challenging them with botulinum toxin A. Twenty μg of toxoid was used per inoculation, as we had previously observed that mice vaccinated twice with only 3μg of BoTd/A in phase one, and then challenged with 5μg (15,000 LD_{50}) of BoNT/A, did not survive (unpublished observation). The usefulness of biopanning to isolate reactive clones is subject to debate. Only 3% of the clones isolated by biopanning against botulinum toxin A were subsequently identified as being reactive to the moderately pure botulinum toxin A.

Due to limited availability of highly purified BoNT/A, moderately pure botulinum A toxin was used in the initial immunoaffinity purification and ELISA of recombinant phage. When reactive clones were subsequently re-assayed using highly purified BoNT/A, it was determined that only 50% of them produced anti-BoNT/A scFv antibodies. The lower purity botulinum toxin A consisted of a complex of bacterial derived "host proteins" and the actual botulinum toxin peptides. Since the lower purity toxoid was used for all animal inoculations, it is likely that many of the scFvs which were nonreactive to the highly pure botulinum toxin A, could be reactive to the "host proteins". An alternative explanation for nonreactivity is that a fairly harsh procedure was used to toxoid the botulinum toxin A, and several of the scFvs could have been reactive only to denatured epitopes. The positive ELISA signals obtained using this highly purified BoNT/A were generally stronger than those obtained using the lower purity BoNT/A. The use of immunoaffinity purification for the selection of clones from scFv antibody libraries provides for a selection based on the binding of scFv antibodies to BoNT/A. ELISA is the preferred methodology for many routine diagnostics because it is rapid and convenient. Accordingly, ELISA was used in further screening reactive clones, thus permitting selection of those clones with sufficiently robust reactivity for use in immunoassay diagnostics. The reliability of ELISA for the identification of the desired phage antibody clones was confirmed using two distinct immunoprecipitation techniques, and further verified by a more direct Western blot analysis.

Our DNA analyses indicated that the inserts in the recombinant phagemid clones were of sufficient size to encode scFv fragments. Deletions arising either from the PCR procedure or from the M13 phage rescue were prominent. Our data suggested no correlation between the reactivity of clones and whether they were intact or truncated. A surprising number of clones were truncated, yet still retained good reactivity to botulinum toxin A. One of the clones, D12 was DNA sequenced (data not shown) and found to have a deletion in the light chain. This is not completely unexpected, as the heavy chain alone has been shown to retain antigen binding (27). This clone will be analyzed further, especially as a model for testing

antibody-antigen reactions by capillary electrophoresis.

Immunoidentification and passive immunotherapeutic techniques are dependant upon the availability of antibodies. Conventional antibodies are produced *in vivo* in animals i.e., polyclonal antibodies, or *in vitro* by culturing of hybridoma cells which secrete monoclonal antibodies. More recently, it has become possible to produce recombinant antibodies e.g., scFv antibodies, through application of the techniques of recombinant DNA technology. For use as immunodiagnostic and immunotherapeutic reagents, recombinant antibodies have a number of potential advantages over conventional antibodies, including enhanced sensitivity, specificity, and reproducibility. In addition, recombinant antibody techniques can be used to rescue important monoclonal antibodies from unstable hybridomas. Of particular importance is the fact that antibody structure and function can be controlled at the gene level through manipulation of genes, resulting in, for example, antibodies with enhanced antigen binding properties, or antibodies fused genetically to other proteins e.g., reporter molecules. Furthermore, "humanized" antibodies which are safe for therapeutic administration to humans can be cloned. Such antibodies can be administered to humans without concern for "serum sickness," resulting from inoculation of animal proteins, or for the presence of adventitious agents, which are portential contaminants of extraneous human immunoglobulins. The use of scFv antibodies also addresses problems of producibility and availability, as the scFv product can be expressed in high volume in alternative hosts and purified therefrom. The yeast system in comparison to bacterial expression systems will be developed, using the D12 clone.

A number of the clones described in this paper were expressed in bacteria. The antibodies produced by three of these clones (A7, D12, 3Ω6) were tested for protection in mice using 10 LD_{50} BoNT/A as challenge, however, none of the antibody lysates were effective in protecting mice (data not shown). The clones and libraries described in this paper are currently undergoing further screening and testing for neutralizing antibodies in collaboration with Dr. R. Titball, (Chemical Biological Defense Establishment, Porton, UK). A major issue which needs to be addressed,

particularly where scFv are to be used therapeutically, is humanization of ScFv antibodies. If neutralizing antibodies are isolated, a second generation chimeric antibody could be engineered to provide protection against BoNT/A. It is technically possible to replace the constant regions of murine monoclonal antibodies with those of human origin, to reduce the "human anti-mouse antibody" effect (25, 26). Experiments to develop chimeric antibodies consisting of scFv of murine origin and constant regions of human origin are currently underway in our laboratories.

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Table I: Summary of mouse scFv to BoNT/A

Clone Description	Estimated scFv gene size (bp)[1]	ELISA with pure BoNT/A[2]	IP with M13[3]	IP with BoNT/A[4]
A4	870	++	ND	ND
A5	780	++	+	++
A7	750	+	++	+
A10	500	+	ND	++
B2	500	+	ND	++
B4	500	++	++	++
B7	500	+++	ND	ND
B8	1300	++	ND	ND
B10	400	++	+	ND
C3	500	++	ND	ND
C10	30	++	++	ND
D1	500	+	+	++
D9	700	++	ND	ND
D10	500	+	++	++
D12	700	++	+++	+
E2	100	+++	ND	ND
E4	600	++	ND	ND
E6	500	++	++	++
E9	500	++	+	+
E10	500	++	ND	ND
E12	150	++	ND	ND
F5	500	++++	ND	ND
F9	200	++	ND	ND
F11	80	++	ND	ND
G1	700	+++	ND	+
G6	900	++	ND	ND
G12	700	++	ND	ND
H4	700	++	ND	ND
H7	700	++	ND	ND

ND=Not Determined

1. Size of scFv genes estimated by restriction digestion with *Sfi* I and *Not* I and/or *Hind* III and *Bam*H I
2. Relative intensity of positive ELISA signals using highly purified BoNT/A
3. Relative intensity of positive autoradiography signals of immunoprecipitated biotinylated BoNT/A using phage antibody
4. Relative intensity of positive autoradiography signals of immunoprecipitated phage antibody using biotinylated BoNT/A

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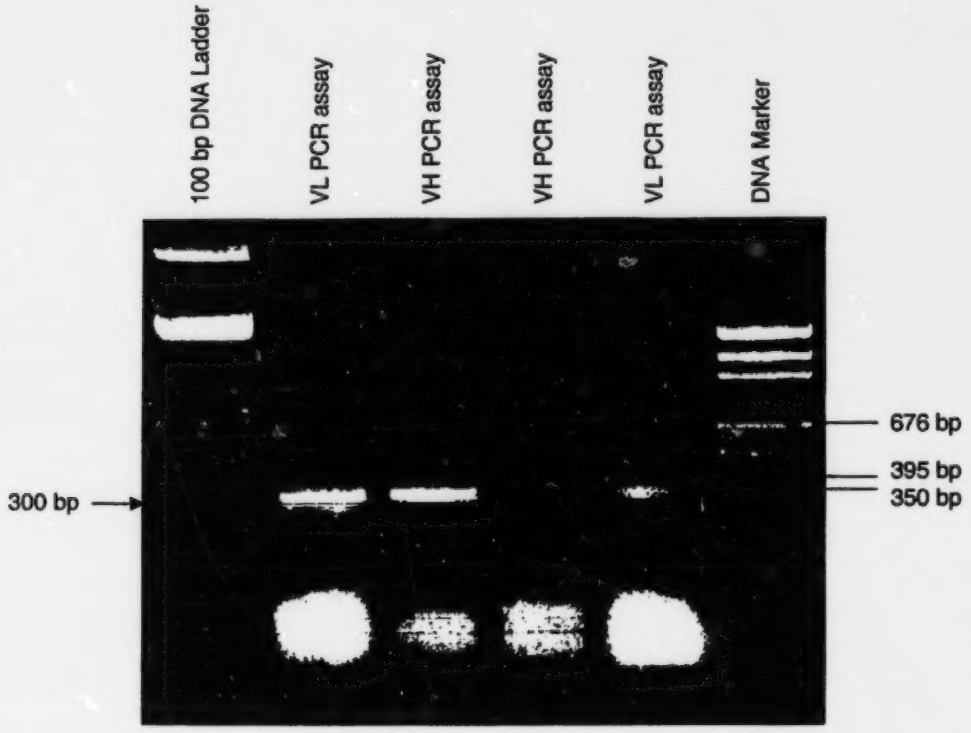

Fig. 1. V_H and V_L DNA fragments, 340 and 325 bp in length, respectively, obtained by PCR amplification of cDNA reverse transcribed from spleen total RNA of BoNT/A-vaccinated Balb/C mice. Typically, 0.1-0.25 μg of cDNA was used for each PCR assay using primer mixtures provided by Pharmacia Biotech. A 100 bp DNA ladder and DNA marker (pGEM, *Hinf* I, *Rsa* I and *Sin* I-digested; Promega, Madison, WI) are included in this 1% agarose gel (1 X TAE buffer). The position of the 300 bp PCR products obtained from PCR amplification using variable light-chain (V_L) primers and variable heavy-chain (V_H) primers are indicated by the arrow on the left.

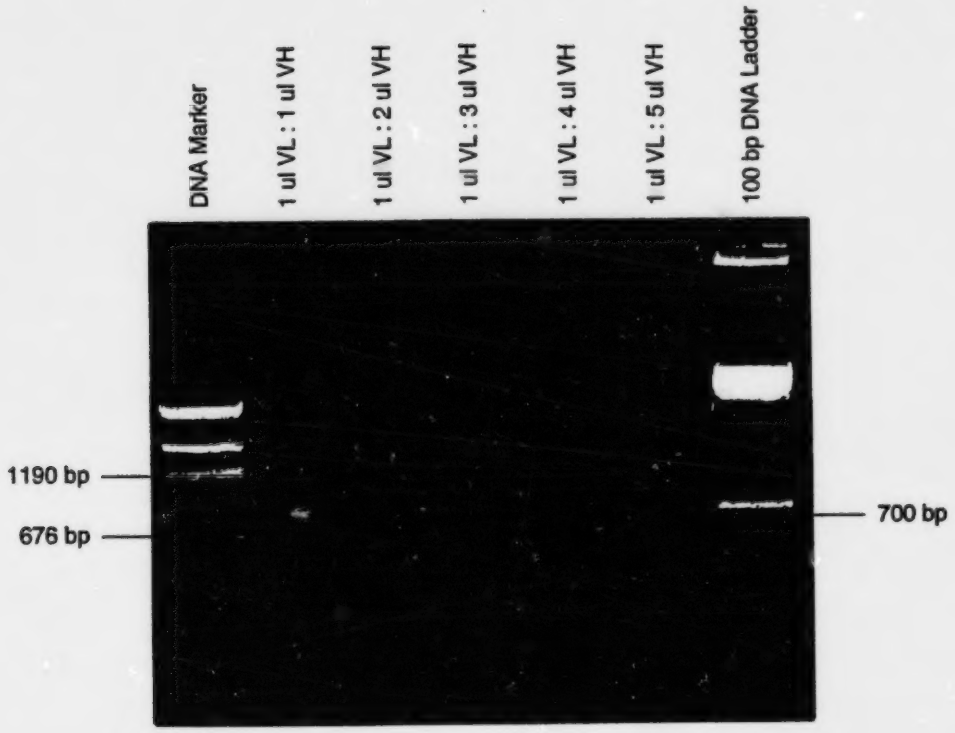

Fig. 2. ScFv DNA fragments, 750 bp in length, obtained by PCR amplification of V_H DNA fragments joined through a linker to V_L DNA fragments. Various ratios of gel-purified V_H DNA:V_L DNA were tested to optimize the amplification reaction. Various amounts of gel-purified V_H and V_L DNA fragments were joined via a linker using *Taq* polymerase. This product was further amplified with the addition of restriction site primers (*Sfi* I and *Not* I) using *Taq* polymerase. The resulting 750 bp fragment was gel-purified and digested with *Sfi* I and *Not* I. The resulting fragments were gel-purified prior to ligation to the pCANTAB 5 cloning vector and transformation into competent TG1 cells.

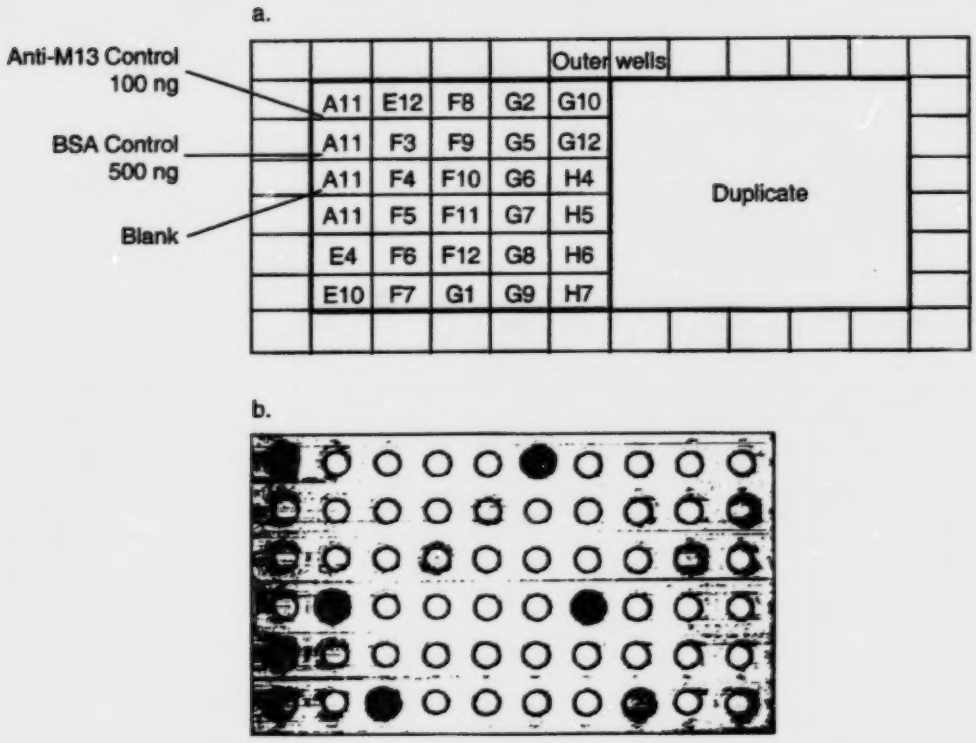

Fig. 3. ELISA of recombinant M13 phage scFv antibody to BoNT/A: a: Sample array on 96-well plates; b: ELISA results. Control antigens (anti-M13 control and BSA) and highly purified BoNT/A (500 ng/well) in 1X coating buffer, pH 9.8, were immobilized on 96-well PVC plates (Costar™). After blocking with 5% skim milk, recombinant M13 phage clones rescued from pCT5 phagemid clones were added to the ELISA plate in duplicate. After 1h of incubation at 37°C, ELISA plates were washed and developed with a 1:2500 dilution of sheep anti-M13 HRP conjugate (Pharmacia Biotech) and ABTS substrate (Kirkegaard and Perry, Gaithersburg, MD). Recombinant M13 clones E12, F5, G1, G6 and G12 display scFv with affinity to purified BoNT/A.

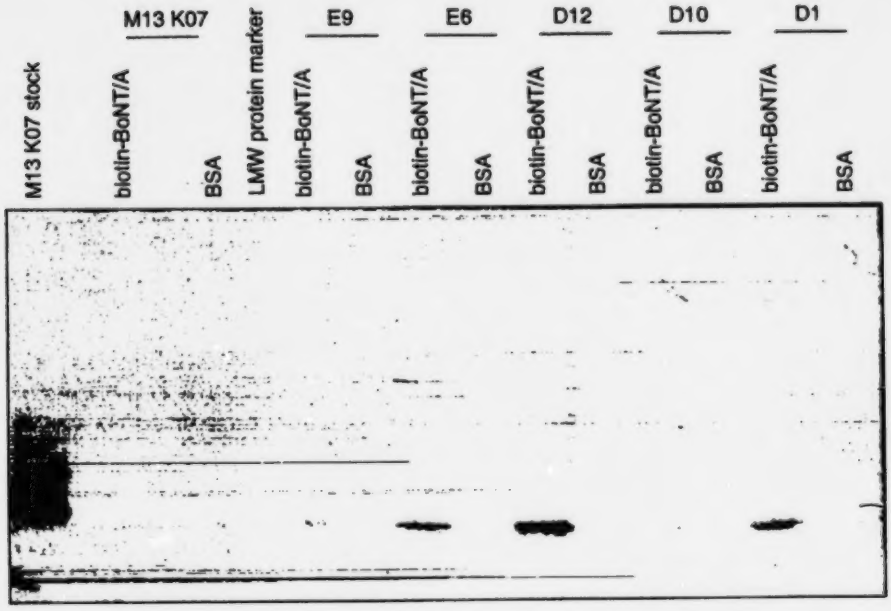

Fig. 4. M13 phage clones incubated with biotinylated BoNT/A and immunoprecipitated with streptavidin beads. M13 phage preparations (20-50 μl) were incubated with either BSA or biotinylated BoNT/A at a final concentration of 10 μgml for 1 h and then with streptavidin beads (20 μl) for 1 h. Beads were washed 3-4 times with Tris-buffered saline (TBS) containing 0.5% Triton X-100 and 0.01% SDS. Proteins were released by incubation in protein loading mix (2% SDS, 2.5% BME, 15% glycerol, 0.2% bromophenol blue) at 100°C for 5 min. Western blots were prepared and immunostained, as previously described. PEG-concentrated preparations of recombinant M13 phage E9, E6, D12, D10 and D1 were incubated with BSA (negative control) or with biotinylated BoNT/A, and then with streptavidin beads. After extensive washes, the immunocomplexes were separated on 12% SDS-PAGE and Western blots were blocked with 5% skim milk, 1% BSA. Blots were immunostained for M13 protein using sheep anti-M13 HRP conjugate and ECL chemiluminescent substrate (Amersham). The LMW prestained protein marker does not immunostain, and was used to monitor electrophoresis. A sample of the M13KO7 helper phage was used as a marker. Immunoprecipitation using M13KO7 served as a negative control for the nonspecific binding of M13 phage to biotinylated BoNT/A.

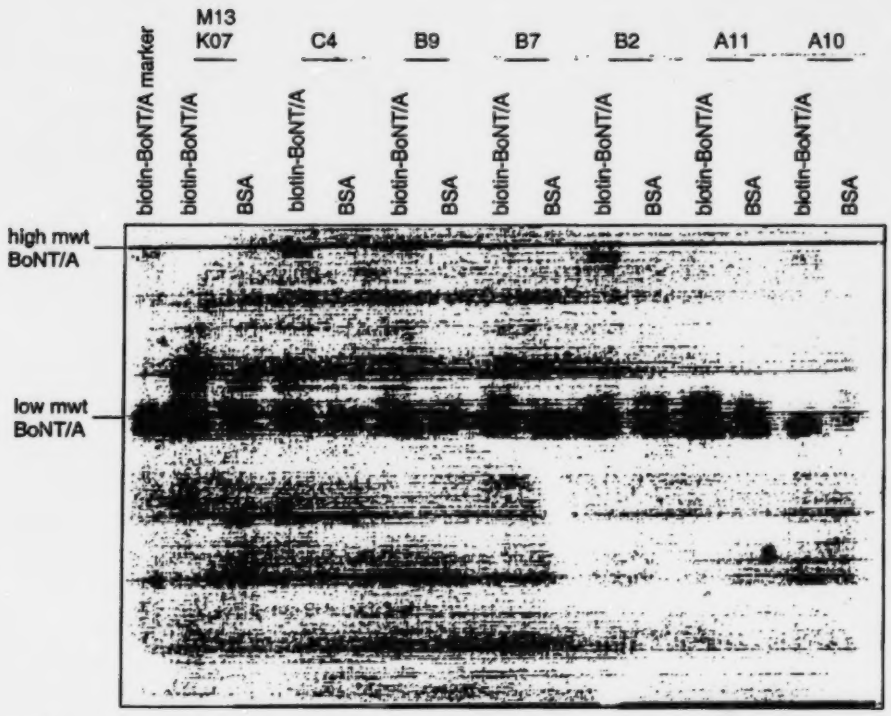

Fig. 5. Biotinylated BoNT/A immunoprecipitated with recombinant M13 phage, rabbit anti-M13 antisera, and protein G immobilized on beads. PEG-concentrated preparations of recombinant M13 phage A10, A11, B2, B7, B9 and C4 were incubated with BSA (negative control) or with biotinylated BoNT/A. After binding to a 1:100 dilution of rabbit anti-M13 antiserum and then to protein G beads, immunocomplexes were extensively washed. Immunocomplexes were separated on 12% SDS-PAGE and Western blots were blocked with 5% skim milk, 1% BSA. Blots were immunostained with biotinylated BoNT/A using streptavidin-alkaline phosphatase conjugate and CSPD chemiluminescent substrate (Stratagene, La Jolla, CA). The biotin-immunoprecipitation using M13KO7 served as a negative control for the nonspecific binding of M13 phage to biotinylated BoNT/A.

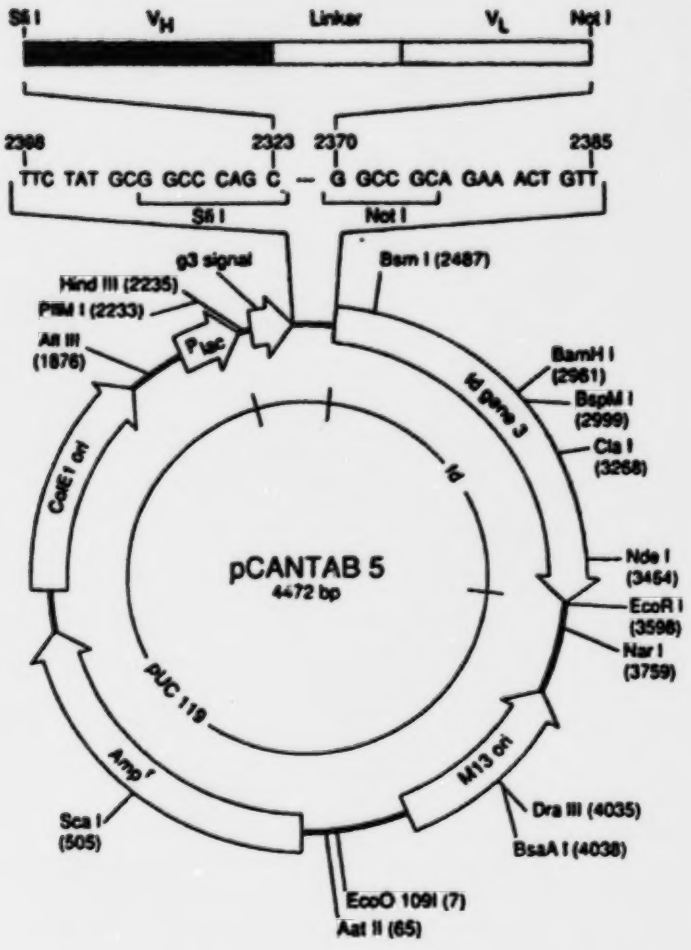

Fig. 6. Restriction map of scFv pCANTAB 5 clones. The diagram shows the restriction sites of recombinant pCANTAB 5 scFv phagemid which were used to estimate gene size of scFv DNA inserts.

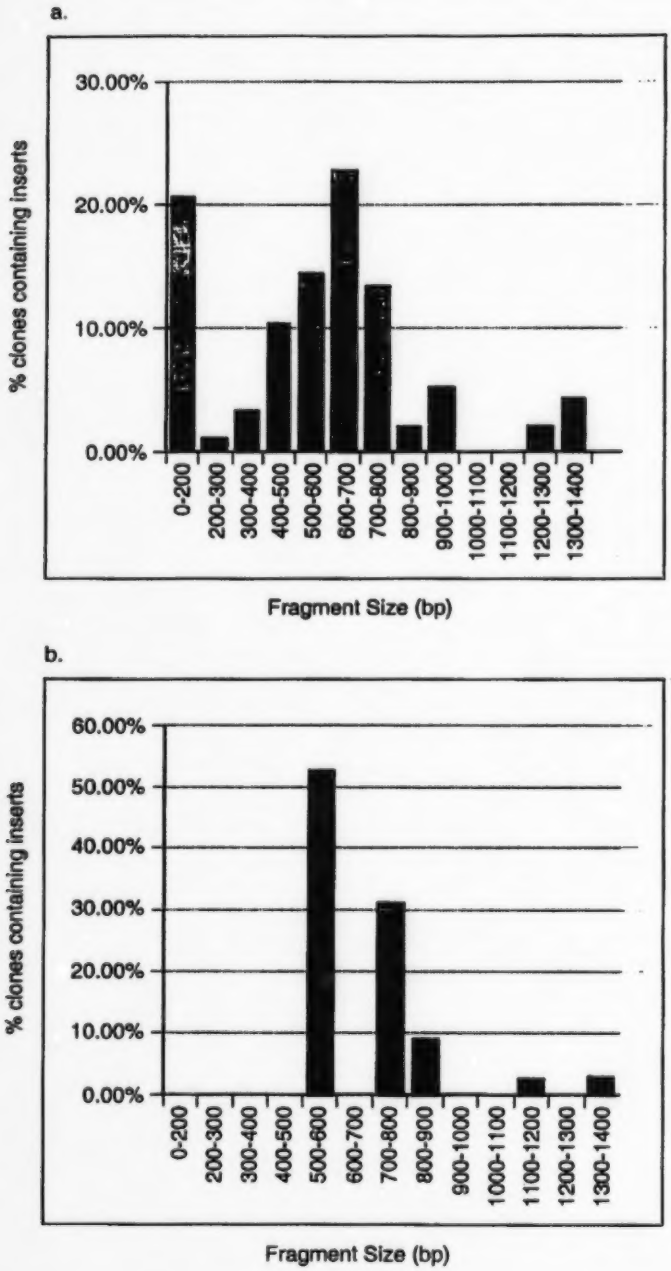

Fig. 7. Size distribution of scFv gene fragments: a: Data compiled from restriction digestion results of 98 of the 131 scFv clones digested with *Hind* III and *BamH* I; b: ScFv fragment size distribution of the 29 most reactive anti-BoNT/A scFv clones digested with *Sfi* I and *Not* I (see Table I).